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From: Chen, Shin-Lin
Sent: Thursday, June 05, 2003 6:47 PM
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Please provide the following articles ASAP. Thanks!
Serial No. 09/788,188.

L4 ANSWER 8 OF 18 CAPLUS COPYRIGHT 2003 ACS DUPLICATE 2
AN 2000:155774 CAPLUS
DN 132:303585
TI Nerve growth factor .alpha. subunit: effect of site-directed mutations on catalytic activity and 7S NGF complex formation
AU Yarski, M. A.; Bax, B. D.; Hogue-Angeletti, R. A.; Bradshaw, R. A.
CS College of Medicine, Department of Physiology and Biophysics, University of California, Irvine, CA, USA
SO Biochimica et Biophysica Acta (2000), 1477(1-2), 253-266.

L4 ANSWER 7 OF 18 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
AN 2000:234365 BIOSIS
DN PREV200000234365
TI Nerve growth factor-induced phosphorylation of SNAP-25 in PC12 cells: A possible involvement in the regulation of SNAP-25 localization.
AU Kataoka, Masakazu; Kuwahara, Reiko; Iwasaki, Satoshi; Shoji-Kasai, Yoko; Takahashi, Masami (1)
CS (1) Mitsubishikasei Institute of Life Sciences, Minami-ooya 11, Machida, Tokyo, 194-8511 Japan
SO Journal of Neurochemistry, (May, 2000) Vol. 74, No. 5, pp. 2058-2066.

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CS College of Medicine, Department of Physiology and Biophysics, University of California, Irvine, CA, USA
SO Biochimica et Biophysica Acta (2000), 1477(1-2), 253-266.

L4 ANSWER 7 OF 18 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
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DN PREV200000234365
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AU Kataoka, Masakazu; Kuwahara, Reiko; Iwasaki, Satoshi; Shoji-Kasai, Yoko; Takahashi, Masami (1)
CS (1) Mitsubishikasei Institute of Life Sciences, Minami-ooya 11, Machida, Tokyo, 194-8511 Japan
SO Journal of Neurochemistry, (May, 2000) Vol. 74, No. 5, pp. 2058-2066.

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=> d his

     (FILE 'HOME' ENTERED AT 18:36:02 ON 05 JUN 2003)

     FILE 'MEDLINE, CAPLUS, BIOSIS, SCISEARCH' ENTERED AT 18:36:12 ON 05 JUN
     2003
L1          900 S (MUTANT OR MUTAT?)(6A)(NEUROTROPHIN OR NGF OR NERVE(W)GROWTH(
L2       297944 S ASPARAGINE OR SERINE
L3           31 S L1 AND L2
L4           18 DUP REM L3 (13 DUPLICATES REMOVED)

=> d bib ab 1-18 l4

L4   ANSWER 1 OF 18  CAPLUS  COPYRIGHT 2003 ACS
AN   2002:658261  CAPLUS
DN   137:196685
TI   Asparagine to serine substitution in N-glycosylation
     site of pro-neurotrophin for improved secretion efficiency and activity
IN   Tuszynski, Mark; Blesch, Armin
PA   Regents of the University of California, USA
SO   PCT Int. Appl., 30 pp.
     CODEN: PIXXD2
DT   Patent
LA   English
FAN.CNT 1
     PATENT NO.        KIND  DATE          APPLICATION NO.  DATE
     --------------    ----  --------      ---------------  --------
PI   WO 2002066645     A2    20020829      WO 2002-US4395   20020215
     WO 2002066645     A3    20030501
         W:  AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN,
             CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM,
             HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS,
             LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO,
             RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN,
             YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
         RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AT, BE, CH,
             CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR,
             BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG
     US 2003040082     A1    20030227      US 2001-788188   20010216
PRAI US 2001-788188    A     20010216
AB   The invention provides mutant nervous system growth factors ("neurotrophic
     factors ") with improved activity.  In particular, the pro-neurotrophins
     of the invention enable a mature neurotrophin protein to be secreted more
     efficiently from host cells than wildtype neurotrophins.  At the higher
     levels of extracellular growth factor achieved by the invention, the
     bioavailability, and therefore the therapeutic potential of the mature
     protein is enhanced.  "Mutant pro-neurotrophins" of
     the invention are produced through substitution of a basic residue, such
     as serine or a similarly charged residue, for the
     asparagine of a targeted N-glycosylation site.  In neurotrophin
     family, the targeted N-glycosylation site is located 4 or 8 amino acids
     upstream of the cleavage site for the corresponding neurotrophin.  This
     site is conserved in all known neurotrophin-family pro-neurotrophins.  As
     such, it is reasonably expected that substitutions made according to the
     invention to all neurotrophin-family pro-neurotrophins will produce
     comparable improvements in secretion efficiency.

L4   ANSWER 2 OF 18     MEDLINE                              DUPLICATE 1
AN   2002473979.     MEDLINE
DN   22206615   PubMed ID: 12114502
TI   Essential role of cAMP-response element-binding protein activation by A2A
     adenosine receptors in rescuing the nerve growth factor-induced neurite
     outgrowth impaired by blockage of the MAPK cascade.
```

AU Cheng Hsiao-Chun; Shih Hsiu-Ming; Chern Yijuang
CS Division of Neuroscience, Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan, Republic of China.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (2002 Sep 13) 277 (37) 33930-42. Journal code: 2985121R. ISSN: 0021-9258.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 200212
ED Entered STN: 20020919
Last Updated on STN: 20030105
Entered Medline: 20021203
AB We found in the present study that stimulation of the A(2A) adenosine receptor (A(2A)-R) using an A(2A)-selective agonist (CGS21680) rescued the blockage of nerve growth factor (NGF)-induced neurite outgrowth when the NGF-evoked MAPK cascade was suppressed by an MEK inhibitor (PD98059) or by a dominant-negative MAPK mutant (dnMAPK). This action of A(2A)-R (designated as the A(2A)-rescue effect) can be blocked by two inhibitors of protein kinase A (PKA) and was absent in a PKA-deficient PC12 variant. Activation of the cAMP/PKA pathway by forskolin exerted the same effect as that by A(2A)-R stimulation. PKA, thus, appears to mediate the A(2A)-rescue effect. Results from cAMP-response element-binding protein (CREB) phosphorylation at **serine** 133, trans-reporting assays, and overexpression of two dominant-negative CREB mutants revealed that A(2A)-R stimulation led to activation of CREB in a PKA-dependent manner and subsequently reversed the damage of NGF-evoked neurite outgrowth by PD98059 or dnMAPK. Expression of an active **mutant** of CREB readily rescued the **NGF**-induced neurite outgrowth impaired by dnMAPK, further strengthening the importance of CREB in the NGF-mediated neurite outgrowth process. Moreover, simultaneous activation of the A(2A)-R/PKA/CREB-mediated and the phosphatidylinositol 3-kinase pathways caused neurite outgrowth that was not suppressed by a selective inhibitor of TrkA, indicating that transactivation of TrkA was not involved. Collectively, CREB functions in conjunction with the phosphatidylinositol 3-kinase pathway to mediate the neurite outgrowth process in PC12 cells.

L4 ANSWER 3 OF 18 CAPLUS COPYRIGHT 2003 ACS
AN 2002:796002 CAPLUS
DN 137:309432
TI Mutations conferring resistance to neutralization by a soluble form of the neurotrophin receptor (p75NTR) map outside of the known antigenic sites of the rabies virus glycoprotein
AU Langevin, Christelle; Tuffereau, Christine
CS Virologie Moleculaire et Structurale, UMR CNRS-INRA 2472, Gif-sur-Yvette, 91198, Fr.
SO Journal of Virology (2002), 76(21), 10756-10765
CODEN: JOVIAM; ISSN: 0022-538X
PB American Society for Microbiology
DT Journal
LA English
AB The neurotrophin receptor (p75NTR) serves as a receptor for rabies virus (RV). The authors expressed and purified a sol. chimera consisting of the p75NTR ectodomain fused to the human IgG1 Fc fragment (p75-Fc). Although p75-Fc interacts with RV, the infectivity of RV did not decrease significantly when it was incubated in the presence of the sol. receptor alone. However, when it was subsequently incubated with an antihuman IgG directed against the Fc fragment of p75-Fc, the infectivity of RV was significantly lowered (>90%), whereas incubation with antihuman IgG alone had no effect. The authors then selected eight independent RV mutants that were not neutralized by p75-Fc and antihuman IgG (srr [sol. receptor resistant] mutants). Each mutant carried a single mutation in the glycoprotein gene leading to one amino acid substitution in the protein. A total of four different substitutions were found. Two of the mutations

were located at position 318 (phenylalanine replaced by a **serine** or a valine residue), and two were located at position 352 (histidine replaced by a tyrosine or an arginine residue). All of the mutations prevented the interaction with p75NTR as either a sol. or a membrane-anchored form. Two mutants (F318S) and (H352R) resulted in the formation of small plaques on BSR cells, probably due to the slower maturation of the glycoprotein. Immunopptn., immunofluorescence, and neutralization assays showed that the four mutated glycoproteins still interacted with representative anti-RV glycoprotein monoclonal antibodies (MAbs), indicating that p75NTR binds outside of the known RV glycoprotein antigenic sites.

RE.CNT 73 THERE ARE 73 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L4 ANSWER 4 OF 18 CAPLUS COPYRIGHT 2003 ACS
AN 2001:413140 CAPLUS
DN 135:150846
TI EGR2 mutations in inherited neuropathies dominant-negatively inhibit myelin gene expression
AU Nagarajan, Rakesh; Svaren, John; Le, Nam; Araki, Toshiyuki; Watson, Mark; Milbrandt, Jeffrey
CS Departments of Pathology and Internal Medicine, Washington University School of Medicine, St. Louis, MO, 63110, USA
SO Neuron (2001), 30(2), 355-368
CODEN: NERNET; ISSN: 0896-6273
PB Cell Press
DT Journal
LA English
AB The identification of EGR2 mutations in patients with neuropathies and the phenotype Egr2/Krox20-/- have demonstrated that the Egr2 transcription factor is crit. for peripheral nerve myelination. However, the mechanism by which these mutations cause disease remains unclear, as most patients present with disease in the heterozygous state, whereas Egr2+/- mice are phenotypically normal. To understand the effect of aberrant Egr2 activity on Schwann cell gene expression, we performed microarray expression profiling to identify genes regulated by Egr2 in Schwann cells. These include genes encoding myelin proteins and enzymes required for synthesis of normal myelin lipids. Using these newly identified targets, we have shown that neuropathy-assocd. EGR2 mutants dominant-neg. inhibit wild-type Egr2-mediated expression of essential myelin genes to levels sufficiently low to result in the abnormal myelination obsd. in these patients.

RE.CNT 68 THERE ARE 68 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L4 ANSWER 5 OF 18 CAPLUS COPYRIGHT 2003 ACS
AN 2000:129299 CAPLUS
DN 132:262026
TI Disruption of the 14-3-3 binding site within the B-Raf kinase domain uncouples catalytic activity from PC12 cell differentiation
AU MacNicol, Melanie C.; Muslin, Anthony J.; MacNicol, Angus M.
CS Department of Medicine, The University of Chicago, Chicago, IL, 60637, USA
SO Journal of Biological Chemistry (2000), 275(6), 3803-3809
CODEN: JBCHA3; ISSN: 0021-9258
PB American Society for Biochemistry and Molecular Biology
DT Journal
LA English
AB A no. of Raf-assocd. proteins have recently been identified, including members of the 14-3-3 family of phosphoserine-binding proteins. Although both pos. and neg. regulatory functions have been ascribed for 14-3-3 interactions with Raf-1, the mechanisms by which 14-3-3 binding modulates Raf activity have not been fully established. We report that mutational disruption of 14-3-3 binding to the B-Raf catalytic domain inhibits B-Raf biol. activity. Expression of the isolated B-Raf catalytic domain (B-Rafcat) induces PC12 cell differentiation in the absence of nerve

growth factor. By contrast, the B-Rafcat 14-3-3 binding mutant, B-Rafcat S728A, was severely compromised for the induction of PC12 cell differentiation. Interestingly, the B-Rafcat 14-3-3 binding mutant retained significant in vitro catalytic activity. In Xenopus oocytes, the analogous full-length B-Raf 14-3-3 binding mutant blocked progesterone-stimulated maturation and the activation of endogenous mitogen-activated protein kinase kinase and mitogen-activated protein kinase. Similarly, the full-length B-Raf 14-3-3 binding **mutant** inhibited **nerve growth factor**-stimulated PC12 cell differentiation. We conclude that 14-3-3 interaction with the catalytic domain is not required for kinase activity per se but is essential to couple B-Raf catalytic activity to downstream effector activation.

RE.CNT 53 THERE ARE 53 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L4 ANSWER 6 OF 18 MEDLINE
AN 2000102732 MEDLINE
DN 20102732 PubMed ID: 10636920
TI Activation of the Stat3 signaling pathway is required for differentiation by interleukin-6 in PC12-E2 cells.
AU Wu Y Y; Bradshaw R A
CS Departments of Physiology and Biophysics, College of Medicine, University of California, Irvine, California 92697, USA.
NC AG09735 (NIA)
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (2000 Jan 21) 275 (3) 2147-56.
Journal code: 2985121R. ISSN: 0021-9258.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 200002
ED Entered STN: 20000309
Last Updated on STN: 20000309
Entered Medline: 20000224
AB The role of signal transducer and activator of transcription (STAT) signaling pathways in the interleukin-6 (IL-6)-induced morphological differentiation of PC12-E2 cells was assessed using wild type and dominant negative mutants of Stat1 and Stat3, containing Tyr --> Phe (YF), Ser --> Ala (SA), and the double mutations (DM), respectively. FS3-YF or FS3-DM markedly inhibited the IL-6-induced response, but overexpression of FS3-SA caused only a modest inhibition. Expression of all Stat3 **mutants** had no effect on **NGF**-induced neurite outgrowth. Overexpression of wild type Stat1 protein inhibited IL-6 activated DNA binding complexes containing Stat3 homodimers, which may explain the partial negative effect of Stat1 on IL-6-induced neurite outgrowth. Specificity of these STAT constructs was confirmed using luciferase reporter gene assays, which showed that IL-6-activated transcription was blocked by expression of FS3-YF and FS3-DM and that FS1 enhanced the interferon gamma-activated transcription. Thus, in PC12-E2 cells, Stat3 homodimers are preferentially activated by IL-6, indicating a role for Stat3 in the regulation of cellular differentiation. Furthermore, IL-6 induced robust neurite outgrowth in PC12-E2 cells expressing dominant negative forms of RAS or SHC or in cells pretreated with the mitogen-activated protein kinase mitogen-activated protein kinase kinase inhibitor, PD98059. Thus, activation of the Stat3 signaling pathway, but not RAS/ERK dependent pathways, is essential for differentiation of PC12-E2 cells by IL-6.

L4 ANSWER 7 OF 18 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
AN 2000:234365 BIOSIS
DN PREV200000234365
TI Nerve growth factor-induced phosphorylation of SNAP-25 in PC12 cells: A possible involvement in the regulation of SNAP-25 localization.
AU Kataoka, Masakazu; Kuwahara, Reiko; Iwasaki, Satoshi; Shoji-Kasai, Yoko;

Takahashi, Masami (1)
CS (1) Mitsubishikasei Institute of Life Sciences, Minami-ooya 11, Machida, Tokyo, 194-8511 Japan
SO Journal of Neurochemistry, (May, 2000) Vol. 74, No. 5, pp. 2058-2066. ISSN: 0022-3042.
DT Article
LA English
SL English
AB Synaptosomal-associated protein of 25 kDa (SNAP-25), a t-SNARE protein essential for neurotransmitter release, is phosphorylated at Ser187 following activation of cellular protein kinase C by treatment with phorbol 12-myristate 12-acetate. However, it remains unclear whether neuronal activity or an endogenous ligand induces the phosphorylation of SNAP-25. Here we studied the phosphorylation of SNAP-25 in PC12 cells using a specific antibody for SNAP-25 phosphorylated at Ser187. A small fraction of SNAP-25 was phosphorylated when cells were grown in the absence of nerve growth factor (NGF). A brief treatment with NGF that was enough to activate the mitogen-activated protein kinase signal transduction pathway did not increase the phosphorylation of SNAP-25; however, phosphorylation was up-regulated after a prolonged incubation with NGF. Up-regulation was transitory, and maximum phosphorylation (a fourfold increase over basal phosphorylation) was achieved between 36 and 48 h after the addition of NGF. Immunofluorescent microscopy showed that SNAP-25 was localized primarily in the plasma membrane, although a significant population was also present in the cytoplasm. Quantitative microfluorometry revealed that prolonged treatment with NGF resulted in a preferential localization of SNAP-25 in the plasma membrane. A mutational study using a fusion protein with green fluorescent protein as a tag indicated that the point **mutation** of Ser187 to Ala abolished the **NGF**-dependent relocalization. A population of SNAP-25 in the plasma membrane was not increased by a point mutation at Ser187 to Glu; however, it was increased by prolonged treatment with NGF, indicating that the SNAP-25 phosphorylation is essential, but not sufficient, for the NGF-induced relocation to the plasma membrane. Our results suggest a close temporal relationship between the up-regulation of SNAP-25 phosphorylation and its relocation, and NGF-induced differentiation of PC12 cells.

L4 ANSWER 8 OF 18 CAPLUS COPYRIGHT 2003 ACS DUPLICATE 2
AN 2000:155774 CAPLUS
DN 132:303585
TI **Nerve growth factor** .alpha. subunit: effect of site-directed **mutations** on catalytic activity and 7S **NGF** complex formation
AU Yarski, M. A.; Bax, B. D.; Hogue-Angeletti, R. A.; Bradshaw, R. A.
CS College of Medicine, Department of Physiology and Biophysics, University of California, Irvine, CA, USA
SO Biochimica et Biophysica Acta (2000), 1477(1-2), 253-266
CODEN: BBACAQ; ISSN: 0006-3002
PB Elsevier Science B.V.
DT Journal
LA English
AB Mouse .alpha.- and .gamma.-nerve growth factor (NGF) are glandular kallikreins that form a non-covalent complex (7S NGF) with .beta.-NGF. .gamma.-NGF is an active arginine-specific esteropeptidase; the .alpha.-subunit is catalytically inactive and has a zymogen-like conformation. Site-directed mutagenesis of .alpha.-NGF to alter the N-terminus and three residues in loop 7, a region that contributes to the catalytic center, restored substantial catalytic activity against N-benzoyl arginine-p-nitroanilide as substrate in two derivs. although they were not as active as recombinant .gamma.-NGF. Seven of the 15 derivs. that remained more .alpha.-like were able to substitute for native .alpha.-NGF in reforming 7S complexes; the other eight derivs. that were more .gamma.-like showed greatly reduced ability to do so. However, the most .gamma.-like .alpha.-NGF deriv. could not substitute for native

.gamma.-NGF in 7S complex formation. These findings suggest that the .alpha.-NGF backbone can be cor. to a functional enzyme by the addn. of a normal N-terminal structure and two catalytic site substitutions and that the 7S complex requires one kallikrein subunit in the zymogen form and one in an active conformation.

RE.CNT 44 THERE ARE 44 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L4 ANSWER 9 OF 18 MEDLINE
AN 1999167336 MEDLINE
DN 99167336 PubMed ID: 10066421
TI Inactivation of mitogen-activated protein kinases by a mammalian tyrosine-specific phosphatase, PTPBR7.
AU Ogata M; Oh-hora M; Kosugi A; Hamaoka T
CS Biomedical Research Center, Osaka University Medical School C6, 2-2 Yamadaoka, Osaka, Suita, 565-0871, Japan.. mogata@ongene.med.osaka-u.ac.jp
SO BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (1999 Mar 5) 256 (1) 52-6.
Journal code: 0372516. ISSN: 0006-291X.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199904
ED Entered STN: 19990426
Last Updated on STN: 20000303
Entered Medline: 19990413
AB Mitogen-activated protein kinase (MAPK) is inactivated through dephosphorylation of tyrosyl and threonyl regulatory sites. In yeast, both dual-specificity and tyrosine-specific phosphatases are involved in dephosphorylation. In mammals, however, no tyrosine-specific phosphatase has been identified molecularly to dephosphorylate MAPK in vivo. Recently, we and others have cloned a murine tyrosine-specific phosphatase, PTPBR7/PTP-SL, which is expressed predominantly in the brain. Here we report inactivation of the extracellular signal-regulated kinase (ERK) family MAPK by PTPBR7. PTPBR7 made complexes with ERK1/ERK2 in vivo and dephosphorylated ERK1 in vitro. When overexpressed in mammalian cells, wild-type PTPBR7 suppressed the phosphorylation and activation of ERK by epidermal growth factor (EGF), **nerve growth factor** (**NGF**), and constitutively active MEK1, a **mutant** MAPK kinase. In contrast, catalytically inactive and ERK-binding-deficient mutants revealed little inhibition on the ERK cascade. These results indicate that PTPBR7 suppresses MAPK directly in vivo.

L4 ANSWER 10 OF 18 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.DUPLICATE 3
AN 1998:169683 BIOSIS
DN PREV199800169683
TI Role of the Jun kinase pathway in the regulation of c-Jun expression and apoptosis in sympathetic Neurons.
AU Eilers, Andreas; Whitfield, Jonathan; Babij, Carol; Rubin, Lee L.; Ham, Jonathan (1)
CS (1) Eisai London Res. Lab., Bernard Katz Build., Univ. Coll. London, Gower St., London WC1E 6BT UK
SO Journal of Neuroscience, (March 1, 1998) Vol. 18, No. 5, pp. 1713-1724. ISSN: 0270-6474.
DT Article
LA English
AB When deprived of nerve growth factor (NGF), developing sympathetic neurons die by apoptosis. This death is associated with an increase in the level of c-Jun protein and is blocked by expression of a c-Jun dominant negative **mutant**. Here we have investigated whether **NGF** withdrawal

activates Jun kinases, a family of stress-activated protein kinases that can stimulate the transcriptional activity of c-Jun by phosphorylating **serines** 63 and 73 in the transactivation domain and which can activate c-Jun gene expression. We found that sympathetic neurons contained high basal levels of Jun kinase activity that increased further after NGF deprivation. In contrast, p38 kinase, another stress-activated protein kinase that can also stimulate c-Jun gene expression, was not activated after NGF withdrawal. Consistent with Jun kinase activation, we found using a phospho-c-Jun-specific antibody that c-Jun was phosphorylated on **serine** 63 after NGF withdrawal. Furthermore, expression of a constitutively active form of MEK kinase 1 (MEKK1), which strongly activates the Jun kinase pathway, increased c-Jun protein levels and c-Jun phosphorylation and induced apoptosis in the presence of NGF. This death could be prevented by co-expression of SEKAL, a dominant negative mutant of SAPK/ERK kinase 1 (SEK1), an activator of Jun kinase that is a target of MEKK1. In contrast, expression of SEKAL alone did not prevent c-Jun expression, increases in c-Jun phosphorylation, or cell death after NGF withdrawal. Thus, activation of Jun kinase and increases in c-Jun phosphorylation and c-Jun protein levels occur at the same time after NGF withdrawal, but c-Jun levels and phosphorylation are regulated by an SEK1-independent pathway.

L4 ANSWER 11 OF 18 MEDLINE
AN 97280743 MEDLINE
DN 97280743 PubMed ID: 9135070
TI Influence of Ras and retinoic acid on nerve growth factor induction of transin gene expression in PC12 cells.
AU Cosgaya J M; Recio J A; Aranda A
CS Instituto de Investigaciones Biomedicas, Consejo Superior de Investigaciones Cientificas, Madrid, Spain.
SO ONCOGENE, (1997 Apr 10) 14 (14) 1687-96.
Journal code: 8711562. ISSN: 0950-9232.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199705
ED Entered STN: 19970609
Last Updated on STN: 20000303
Entered Medline: 19970523
AB Nerve growth factor (NGF)- and ras-induced neuronal differentiation of PC12 cells is accompanied by expression of transin, a secreted metalloproteinase. Retinoic acid (RA) is known to exert important effects on neural cell proliferation and differentiation. In this study we have analysed different PC12 sublines which express either activated Ras or dominant negative p21N17 Ras, to evaluate the influence of retinoic acid (RA) on the response of the transin gene to NGF and Ras. There was a good correlation between neurite extension and induction of transin mRNA levels in the different subclones. NGF did not induce transin mRNA in cells which do not differentiate in response to this neurotrophin. In addition, incubation with RA did not detectably increase basal transin mRNA levels, but caused a significant increase in the transin response to NGF or Ras in cells in which these factors induce a neuronal morphology. Sequences contained within 750 base pairs of the 5' flanking region of the transin gene confer responsiveness to NGF and Ras, but do not mediate the stimulatory effect of RA. In addition, expression of oncogenic Raf increases transin promoter activity in PC12 cells, but a dominant-negative Raf **mutant** was unable to block **NGF**-induced transin activity suggesting the existence of a bifurcation downstream of ras in the signaling mechanism leading to transin expression by NGF.

L4 ANSWER 12 OF 18 MEDLINE DUPLICATE 4
AN 97467004 MEDLINE
DN 97467004 PubMed ID: 9328344

TI Differential regulation of mitogen-activated protein/ERK kinase (MEK)1 and MEK2 and activation by a Ras-independent mechanism.
AU Xu S; Khoo S; Dang A; Witt S; Do V; Zhen E; Schaefer E M; Cobb M H
CS University of Texas Southwestern Medical Center, Department of Pharmacology, Dallas 75235-9041, USA.
NC DK-34128 (NIDDK)
SO MOLECULAR ENDOCRINOLOGY, (1997 Oct) 11 (11) 1618-25.
Journal code: 8801431. ISSN: 0888-8809.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199801
ED Entered STN: 19980129
Last Updated on STN: 20000303
Entered Medline: 19980112
AB Mitogen-activated protein (MAP)/ERK kinase (MEK)1 and MEK2 are the upstream activators of the MAP kinases, ERK1 and ERK2. MEK1 and MEK2 are approximately 85% identical in sequence but have unique inserts in their C-terminal domains. MEK isoform-specific antibodies were used to examine expression and regulation of each enzyme. MEK1 and MEK2 were expressed in approximately equal amounts in several cell lines; in some, MEK1 was present in slight excess. Activation of tyrosine kinase-containing receptors, heterotrimeric G proteins, and protein kinase C enhanced the activities of both MEK isoforms in 293 and PC12 cells. AIF4-stimulated both MEK1 and MEK2 in PC12 cells expressing a dominant interfering Ras **mutant** that prevents **nerve growth factor**-dependent activation of the cascade. Carbachol also stimulated the pathway in these cells. Thus, in addition to their ability to activate Ras/Raf and the downstream ERK pathway, heterotrimeric G proteins also appear to trigger a Ras-independent mechanism to regulate this kinase cascade. In U373, Chinese hamster ovary (CHO), and INS-1 cells, MEK1 was activated by regulators of ERKs, while MEK2 was not. These data suggest that, like the MAP kinases ERK1 and ERK2, in some cell settings the two similar MEK isoforms are differentially regulated.

L4 ANSWER 13 OF 18 MEDLINE
AN 95036335 MEDLINE
DN 95036335 PubMed ID: 7524763
TI Poor response of cultured mast cells derived from mi/mi **mutant** mice to **nerve growth factor**.
AU Jippo T; Ushio H; Hirota S; Mizuno H; Yamatodani A; Nomura S; Matsuda H; Kitamura Y
CS Department of Pathology, Osaka University Medical School, Japan.
SO BLOOD, (1994 Nov 1) 84 (9) 2977-83.
Journal code: 7603509. ISSN: 0006-4971.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 199411
ED Entered STN: 19950110
Last Updated on STN: 20021022
Entered Medline: 19941129
AB Decreased numbers of mast cells and abnormalities in the phenotype of mast cells are observed in the skin of mi/mi mutant mice. Recently, the mi locus was identified to encode a novel member of the basic-helix-loop-helix-leucine zipper protein family of transcription factors. Since nerve growth factor (NGF) has been reported to influence the proliferation and the phenotype of cultured mast cells (CMCs), we compared the effect of NGF between mi/mi and control normal (+/+) CMCs. Addition of NGF to the suboptimal dose of recombinant murine interleukin-3 (rmIL-3) increased the plating efficiency of +/+ CMCs, but not of mi/mi CMCs. Although +/+ CMCs were berberine sulfate-negative when cultured with rmIL-3 alone, +/+ CMCs

became berberine sulfate-positive when cultured in the presence of both rmIL-3 and NGF, which suggests increased heparin content. In contrast, NGF did not influence the phenotype of mi/mi CMCs. +/+ CMCs significantly bound 125I-NGF, but mi/mi CMCs did not, which suggests a defect of NGF receptors in mi/mi CMCs. Both p75 and p140 molecules are known to be involved in the formation of NGF receptors. Although the expression of p140 messenger (m)RNA was comparable between +/+ and mi/mi CMCs, the expression of p75 mRNA was significantly lower in mi/mi CMCs than in +/+ CMCs. Taken together, the poor response of mi/mi CMCs to NGF appeared to be attributable to the impaired transcription of the p75 gene.

L4 ANSWER 14 OF 18 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.DUPLICATE 5
AN 1994:255621 BIOSIS
DN PREV199497268621
TI Identification of the sites in MAP kinase kinase-1 phosphorylated by p74-raf-1.
AU Alessi, Dario R.; Saito, Yuji; Campbell, David G.; Cohen, Philip; Sithanandam, Gunamani; Rapp, Ulf; Ashworth, Alan; Marshall, Chris J.; Cowley, Sally (1)
CS (1) Chester Beatty Lab., Inst. Cancer Res., Fulham Road, London SW3 6JB UK
SO EMBO (European Molecular Biology Organization) Journal, (1994) Vol. 13, No. 7, pp. 1610-1619.
ISSN: 0261-4189.
DT Article
LA English
AB Many growth factors whose receptors are protein tyrosine kinases stimulate the MAP kinase pathway by activating first the GTP-binding protein Ras and then the protein kinase p74-raf-1. p74-raf-1 phosphorylates and activates MAP kinase kinase (MAPKK). To understand the mechanism of activation of MAPKK, we have identified Ser217 and Ser221 of MAPKK1 as the sites phosphorylated by p74-raf-1. This represents the first characterization of sites phosphorylated by this proto-oncogene product. Ser217 and Ser221 lie in a region of the catalytic domain where the activating phosphorylation sites of several other protein kinases are located. Among MAPKK family members, this region is the most conserved, suggesting that all members of the family are activated by the phosphorylation of these sites. A 'kinase-dead' MAPKK1 mutant was phosphorylated at the same residues as the wild-type enzyme, establishing that both sites are phosphorylated directly by p74-raf-1, and not by autophosphorylation. Only the diphosphorylated form of MAPKK1 (phosphorylated at both Ser217 and Ser221) was detected, even when the stoichiometry of phosphorylation by p74-raf-1 was low, indicating that phosphorylation of one of these sites is rate limiting, phosphorylation of the second then occurring extremely rapidly. Ser217 and Ser221 were both phosphorylated in vivo within minutes when PC12 cells were stimulated with **nerve growth factor**.
Analysis of MAPKK1 **mutants** in which either Ser217 or Ser221 were changed to glutamic acid, and the finding that inactivation of maximally activated MAPKK1 required the dephosphorylation of both **serines**, shows that phosphorylation of either residue is sufficient for maximal activation.

L4 ANSWER 15 OF 18 SCISEARCH COPYRIGHT 2003 THOMSON ISI
AN 94:159595 SCISEARCH
GA The Genuine Article (R) Number: NC769
TI TRK RECEPTORS USE REDUNDANT SIGNAL-TRANSDUCTION PATHWAYS INVOLVING SHC AND PLC-GAMMA-1 TO MEDIATE NGF RESPONSES
AU STEPHENS R M (Reprint); LOEB D M; COPELAND T D; PAWSON T; GREENE L A; KAPLAN D R
CS NCI, FREDERICK CANC RES & DEV CTR, ABL BASIC RES PROGRAM, FREDERICK, MD, 21702 (Reprint); COLUMBIA UNIV, DEPT PATHOL, NEW YORK, NY, 10032; COLUMBIA UNIV, CTR NEUROBIOL & BEHAV, NEW YORK, NY, 10032; UNIV TORONTO, DEPT MOLEC & MED GENET, TORONTO, ON, CANADA
CYA USA; CANADA

SO NEURON, (MAR 1994) Vol. 12, No. 3, pp. 691-705.
ISSN: 0896-6273.
DT Article; Journal
FS LIFE
LA ENGLISH
REC Reference Count: 83
ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS
AB In response to NCF, the Trk receptor tyrosine kinase forms a complex with SHC, a protein that couples receptor tyrosine kinases to p21(ras). Complex formation between Trk and SHC, SHC tyrosine phosphorylation, and association of SHC with Grb2 were mediated by autophosphorylation at Y490 in Trk (NPQYFSD). To determine the role of SHC and other Trk substrates in **NGF** signaling, Trk receptors with **mutations** in Y490 and Y785 (the PLC-gamma 1 association site) were introduced into PC12nnr5 cells. NGF treatment of PC12nnr5 cells expressing Trk with mutations in either substrate-binding site resulted in normal neurite outgrowth and Erk1 activity and tyrosine phosphorylation. However, PC12nnr5 cells expressing Trk with mutations at both sites failed to stably extend neurites and efficiently induce Erk1 activity and tyrosine phosphorylation in response to NGF. We postulate that Trk receptors can activate Erk1 by either SHC- or PLC-gamma 1-dependent signaling pathways. These results suggest a model whereby Trk receptors utilize at least partially redundant signal transduction pathways to mediate NGF responses.

L4 ANSWER 16 OF 18 MEDLINE DUPLICATE 6
AN 94004459 MEDLINE
DN 94004459 PubMed ID: 8401208
TI Prediction of the three-dimensional structures of the nerve growth factor and epidermal growth factor binding proteins (kallikreins) and an hypothetical structure of the high molecular weight complex of epidermal growth factor with its binding protein.
AU Bax B; Blaber M; Ferguson G; Sternberg M J; Walls P H
CS Department of Crystallography, Birkbeck College, London, United Kingdom.
SO PROTEIN SCIENCE, (1993 Aug) 2 (8) 1229-41.
Journal code: 9211750. ISSN: 0961-8368.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199310
ED Entered STN: 19940117
Last Updated on STN: 20000303
Entered Medline: 19931028
AB We have predicted the three-dimensional structures of the **serine** protease subunits (gamma-NGF, alpha-NGF, and EGF-BP) of the high molecular weight complexes of nerve growth factor (7S NGF) and epidermal growth factor (HMW-EGF) from the mouse submandibular gland (from the X-ray crystal structures of two related glandular kallikreins). The conformations of three of the six loops surrounding the active site are relatively well defined in the models of gamma-NGF and EGF-BP, but three other loops are likely to have flexible conformations. Although the amino acid sequence of alpha-NGF is closely related to those of gamma-NGF and EGF-BP, it is catalytically inactive. Model-building studies on alpha-**NGF** suggested that **mutations** (in alpha-**NGF**) just prior to the active site **serine** (195) and an unusual N-terminal sequence are consistent with alpha-NGF having a zymogen-like conformation (similar to that in chymotrypsinogen). An hypothetical model of the quaternary structure of HMW-EGF has been constructed using this model of EGF-BP and the NMR structure of murine EGF. The C-terminal arm of EGF was modeled into the active site of EGF-BP based on data indicating that the C-terminal arginine of EGF occupies the S1 subsite of EGF-BP. Data suggesting one of the surface loops of EGF-BP is buried in the HMW-EGF complex and symmetry constraints were important in deriving a schematic model. A molecular docking program was used to fit EGF to

EGF-BP.

L4 ANSWER 17 OF 18 MEDLINE DUPLICATE 7
AN 92150422 MEDLINE
DN 92150422 PubMed ID: 1686097
TI **Mutation** of tryptophan-21 in mouse **nerve growth factor** (NGF) affects binding to the fast NGF receptor but not induction of neurites on PC12 cells.
AU Drinkwater C C; Suter U; Angst C; Shooter E M
CS Department of Neurobiology, Stanford University School of Medicine, California 94305-5401.
NC NS 04270 (NINDS)
SO PROCEEDINGS OF THE ROYAL SOCIETY OF LONDON. SERIES B: BIOLOGICAL SCIENCES, (1991 Dec 23) 246 (1317) 307-13.
Journal code: 7505889. ISSN: 0962-8452.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199203
ED Entered STN: 19920405
Last Updated on STN: 19980206
Entered Medline: 19920313
AB By using in vitro DNA mutagenesis, we replaced the tryptophan residue at position 21 in mouse nerve growth factor (NGF) with either phenylalanine, leucine or **serine**. Yield, biological activity, immunological reactivity and receptor binding of the recombinant proteins were determined. All three mutants were produced at considerably lower yields than wild-type **NGF**, with the **serine mutant** being undetectable. The results of competitive binding assays show that tryptophan-21 is involved in recognition of the fast NGF receptor of PC12 cells. However, specific biological activity of NGF is not altered by the replacement of tryptophan-21. Our results therefore suggest that biological activity of NGF is not directly coupled to binding to the fast NGF receptor.

L4 ANSWER 18 OF 18 CAPLUS COPYRIGHT 2003 ACS
AN 1990:400665 CAPLUS
DN 113:665
TI Structure-function studies of nerve growth factor: functional importance of highly conserved amino acid residues
AU Ibanez, Carlos F.; Hallboeoek, Finn; Ebendal, Ted; Persson, Haakan
CS Dep. Med. Chem. II, Karolinska Inst., Stockholm, Swed.
SO EMBO Journal (1990), 9(5), 1477-83
CODEN: EMJODG; ISSN: 0261-4189
DT Journal
LA English
AB Selected amino acid residues in chicken nerve growth factor (NGF) were replaced by site-directed mutagenesis. **Mutated NGF** sequences were transiently expressed in COS cells and the yield of NGF protein in conditioned medium was quantified by Western blotting. Binding of each **mutant** to **NGF** receptors on PC12 cells was evaluated in a competition assay. The biol. activity was detd. by measuring stimulation of neurite outgrowth from chick sympathetic ganglia. The residues homologous to the proposed receptor binding site of insulin (**serine**-18, methionine-19, valine-21, and aspartic acid-23) were substituted by alanine. Replacement of **serine**-18, methionine-19, and aspartic acid-23 did not affect NGF activity. Modification of valine-21 notably reduced both receptor binding and biol. activity, suggesting that this residue is important to retain a fully active NGF. The highly conserved tyrosine-51 and arginine-99 were converted into phenylalanine and lysine, resp., without changing the biol. properties of the mol. However, binding and biol. activity were greatly impaired after the simultaneous replacement of both arginine-102 and

arginine-99 by glycine. The 3 conserved tryptophan residues at positions 20, 75 and 98 were substituted by phenylalanine. The tryptophan mutated proteins retained 15-60% of receptor binding and 40-80% of biol. activity, indicating that the tryptophan residues are not essential for NGF activity. However, replacement of tryptophan-20 reduced the amt. of NGF in the medium, suggesting that this residue may be important for protein stability.

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l1 near7 (asparagine or serine)	1

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Search: L6

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L6	l1 near7 (asparagine or serine)	1	L6
L5	l1L4	7	L5
L4	l1 with l2	1	L4
L3	l1 and L2	84	L3
L2	asparagine or serine	41373	L2
L1	(mutant or mutat$) near6 (neurotrophin or ngf or nerve adj growth adj factor)	125	L1

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☐ 1. 20030040082 . 16 Feb 01. 27 Feb 03. Mutant pro-neurotrophin with improved activity. Tuszynski, Mark, et al. 435/69.4; 424/93.21 435/320.1 435/325 530/399 536/23.5 A61K048/00 C07H021/04 C12N005/06 A61K038/18 C07K014/48.

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Volume 271, Number 41, Issue of October 11, 1996 pp. 25430-25437

The Regulated Secretion and Vectorial Targeting of Neurotrophins in Neuroendocrine and Epithelial Cells*

(Received for publication, May 24, 1996, and in revised form, July 5, 1996)

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ABSTRACT

The varied roles that neurotrophins play in the development and activity-dependent plasticity of the nervous system presumably require that the sites and quantity of neurotrophin release be precisely regulated. As a step toward understanding how different neurotrophins are sorted and secreted by neurons, we expressed nerve growth factor (NGF), brain-derived neurotrophic factor, and neurotrophin-3 in cell lines used as models for neuronal protein sorting. All three neurotrophins were secreted by a regulated pathway in transfected AtT-20 and PC12 neuroendocrine cells, with a 3-6-fold increase in neurotrophin release in response to 8-bromo-cAMP or depolarization, respectively. To determine if the propeptide directs the intracellular sorting of mature NGF, we examined mutants in which regions spanning the propeptide were deleted. These mutants underwent regulated release in every case in which expression could be detected. Similarly, NGF sorting was not significantly altered by mutations which specifically abolished *N*-glycosylation or proteolytic processing sites within the NGF precursor. Finally,

we found that all three neurotrophins were secreted 65-75% basolaterally by polarized Madin-Darby canine kidney epithelial cells. These findings suggest that the determinants of regulated neurotrophin secretion lie within the mature neurotrophin moiety and that NGF, brain-derived neurotrophic factor, and neurotrophin-3 are likely to be sorted similarly and released in a regulated manner by neurons.

INTRODUCTION

NGF,[1] BDNF, NT-3, and NT-4/5 constitute the neurotrophins, a family of structurally related, dimeric proteins that play a critical role in the survival and differentiation of specific neuronal populations in the developing and adult nervous systems (1, 2, 3). Neurotrophins have traditionally been considered in the context of the ``neurotrophic hypothesis,'' which holds that neurons compete for limiting amounts of factors secreted by the target tissue, with only the successful competitors surviving and establishing functional connections (4). In the periphery, (non-neuronal) target tissues are believed to secrete neurotrophins constitutively and regulate neurotrophin mRNA independent of neuronal input (5, 6). Recent studies, however, have unveiled additional complexities in how neurotrophins function. For example, neurotrophins have been implicated in a variety of activity-dependent processes in the central nervous system (reviewed in Refs. 7 an 8) including ocular dominance column formation (9), activity-dependent survival of cortical neurons (10), axonal sprouting, and the long term enhancement of synaptic transmission (11). These studies suggest that the expression and/or release of neurotrophins may be regulated by activity. Furthermore, autocrine or paracrine, as opposed to target-derived, interactions appear to promote the survival of some mature neurons (10, 12, 13), and neurotrophins may also be transported in anterograde direction and participate in neuron to target signaling (14, 15, 16). Since neurons are polarized cells that can target proteins to at least two distinct domains (the axon and somatodendritic domain) and can secrete proteins in either a regulated or constitutive manner, understanding how neurotrophins function requires an understanding of how the neurotrophins are intracellularly sorted and secreted.

The neurotrophins are initially synthesized as precursors containing an amino-terminal propeptide that is proteolytically processed to release the mature neurotrophin. In the case of NGF, this propeptide is glycosylated (17, 18), contains multiple potential cleavage sites, and is required for the proper folding of mature NGF (18). Because neurotrophins are normally expressed at extremely low levels, little is known about how they are sorted and secreted *in vivo* by neurons and other cell types. When heterologously expressed in AtT-20 neuroendocrine cells, in human insuloma (HIT) cells (17), or in hippocampal neurons (19), NGF was found to be secreted via a regulated secretory pathway. Despite these advances, however, fundamental questions about neurotrophin sorting and secretion remain unanswered. For example: are all members of the neurotrophin family released via a regulated secretory pathway? Are there sorting differences between members of the neurotrophin family which are functionally significant, as there are in the platlet-derived growth factor and fibroblast growth factor families (20, 21)? Finally, what regions or motifs within the neurotrophin precursor play a role in directing the neurotrophins to specific secretory pathways or subcellular domains?

To address these questions, we have analyzed neurotrophin secretion in cell lines that are well established model systems for the study of neuronal secretion and targeting. The rat pheochromocytoma PC12 cell line and the mouse pituitary AtT-20 cell line sort proteins into the regulated secretory pathway (22) and possess dense core granules that are similar to neuronal large dense core vesicles in terms of size, morphology, and composition (reviewed in Refs. 23 and 24). PC12 cells, but apparently not AtT-20 cells, also contain synaptic-like microvesicles which resembles neuronal synaptic vesicles (25). Both cell lines share a number of other characteristics with neurons such as electrically excitable membranes and

the ability to extend neurites with growth cones (26, 27). In order to investigate the vectorial targeting of neurotrophins in polarized cells, MDCK cells were used. This is an epithelial cell line which targets surface-bound proteins to one of two distinct domains, the apical and basolateral membranes, by a mechanism which is likely to be, in at least some cases, similar to that used by neurons to sort proteins to axons and dendrites, respectively (28, 29).

In this study we have used transient and stable transfection of NGF, BDNF, and NT-3 to compare the intracellular sorting of the different neurotrophins in AtT-20 and PC12 cells. We have also used site-directed mutagenesis to investigate the role of the NGF propeptide and to determine if mechanisms that are known to influence protein sorting such as *N*-linked glycosylation (30), proteolytic processing (31, 32), or alternative splicing (33, 34) play a role in neurotrophin sorting. We show that NGF, BDNF, and NT-3 are all sorted into the regulated pathway in both types of neuroendocrine cells. Furthermore, this targeting does not require *N*-glycosylation or proteolytic processing of the NGF propeptide and is not affected by deletions scanning most of the propeptide. These findings suggest that different neurotrophins are sorted and secreted similarly, and information required for this sorting is likely to lie within the mature neurotrophin rather than the propeptide.

MATERIALS AND METHODS

Expression Plasmids

The cDNA for mouse NGF, rat BDNF, and rat NT-3 were all subcloned into pBJ-5, an SRα-based expression plasmid (35), as described previously (18, 36). NGF precursor mutants were constructed by the polymerase chain reaction-based overlap extension method, and the coding region of all mutants was sequenced by the dideoxynucleotide method to ensure that errors were not introduced during the polymerase chain reaction process. The construction of the R(−4)Q, N(−8)Q, and deletion mutants, as well as their expression in COS cells, have been previously described (18, 36).

Cell Culture and Transfection

COS-7 cells and PC12 cells were grown in DMEM supplemented with 6% horse serum, 6% bovine calf serum (Hyclone). AtT-20-F2 cells (obtained from A. Lowe) and MDCK type II cells (J clone, obtained from W. J. Nelson) were maintained in DMEM supplemented with 10% fetal calf serum (Life Technologies, Inc.). Penicillin and streptomycin (Life Technologies, Inc.) were added to both media. COS cells were transfected and metabolically labeled for 3 h with 100 μCi of [^{35}S]cysteine (Amersham Corp.) as described previously (18). AtT-20 were stably transfected as described previously (22) with the following modifications: 80 μg of expression plasmid and 20 μg of SV2-neo were used to transfect 1×10^6 cells by the calcium phosphate method (Specialty Media transfection kit). Neurotrophin-expressing clones were selected using cloning cylinders and screened by ELISA.

Transient transfections of AtT-20, PC12, and MDCK cells were performed using LipofectAMINE (Life Technologies, Inc.) as indicated in manufacturer's instructions. The following amounts of expression plasmid DNA and LipofectAMINE were used to transfect 2-3 × 10^6 cells/10-cm plate: for AtT-20 cells, 25 μg of DNA and 100 μl of LipofectAMINE; for PC12 cells, 12 μg of DNA and 75 μl of reagent; for MDCK cells, 5 μg of DNA and 15 μl of LipofectAMINE. COS cells were transiently transfected using the DEAE-dextran/chloroquine method as described previously (18).

AtT-20 and PC12 Secretion Studies

AtT-20 cells were removed from a single 10-cm plate by trypsinization 24 h post-transfection, equally distributed to the wells of a six-well plate, and allowed to grow for an additional 40-48 h in full medium. Cells were then washed twice with DMEM, and three wells were incubated in DMEM, 0.2% BSA (unstimulated controls), while the other three were stimulated by incubation in DMEM, 0.2% BSA with 5 mM 8-Br-cAMP (Sigma) added. Conditioned medium was removed after 6 h and cleared of cellular debris by centrifugation; in some experiments the cells were washed with cold phosphate-buffered saline and lysed in phosphate-buffered saline buffer containing 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.2% BSA, 1 mM EDTA, and protease inhibitors. Neurotrophin content of conditioned medium or lysate was measured by ELISA as described previously (36), and the results from the three stimulated or unstimulated samples were averaged. Control experiments using purified NGF standards indicated that the use of lysis buffer did not appreciably alter the amount of NGF detectable by ELISA. For cycloheximide (CHX) and actinomycin D experiments, the transfected cells were washed and incubated in DMEM, 0.2% BSA containing 100 μg/ml CHX or 10 mM actinomycin D for 1.5 h to inhibit new protein synthesis. Cells were then washed again and incubated for 4 h in the same medium as in the pretreatment. Media and cell lysates were subsequently collected and assayed as described above.

Neurotrophin secretion by transiently transfected PC12 cells was analyzed in a similar manner with the following modifications. Since PC12 cells differentiate in the presence of NGF, 10 ng/ml purified mouse 2.5S NGF (Harlan Bioproducts) was added the medium for all cells 8 h post-transfection; this caused mock-transfected or NT-3-expressing cells to extend neurites to a similar extent as NGF-expressing cells. 72 h post-transfection the NGF-containing medium was removed, and the cells were washed three times with DMEM. Depolarization-induced secretion was then stimulated by adding 50 mM KCl to DMEM, 0.2% BSA medium during the 15-min incubation. Similar results were obtained when modified Hanks' buffer (125 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 μm $ZnCl_2$, 10 mM glucose, 25 mM HEPES, 0.2% BSA, pH 7.4) instead of DMEM was used, and depolarization was induced in modified Hanks' buffer containing 50 mM KCl and 80 mM NaCl.

MDCK Secretion Studies

2.5×10^6 MDCK cells were seeded and grown on 24.5-mm collagen-coated Transwell filters (Costar; 0.4-μm pore size) as described previously (37, 38) with changes of media every day. In general, for each neurotrophin-expressing cell line studied, four filters were seeded in parallel. Three days after seeding, one filter was tested for the presence of functional tight junctions by measuring the diffusion of [^{3}H]inulin (DuPont NEN) from the apical to basolateral compartment for 1 h; filters were used only if the monolayer inhibited greater than 99% of the inulin diffusion. Medium from the other three filters was changed, collected after an additional 24-h incubation, and assayed by ELISA. The percent of neurotrophin secreted into the apical and basolateral compartments was determined for each individual filter, and results from the three filters were averaged. Filter-grown cells were metabolically labeled by incubating for 1 h in labeling medium (DMEM lacking cysteine with 1% dialyzed fetal calf serum added), then adding 250 μCi of [^{35}S]cysteine in labeling medium to the basolateral compartment. After 3-h continuous labeling, the conditioned medium was withdrawn, immunoprecipitated with NGF antiserum, and analyzed by SDS-PAGE. gp81 (clusterin) (39, 40) was detected as described previously (38).

Transient transfection experiments were performed as follows: 3×10^6 cells in 1 ml of serum-free DMEM was combined with a 200-μl DNA/LipofectAMINE mixture (see above) and seeded on filters. After 6-8 h, the medium was replaced with DMEM supplemented with 10% fetal calf serum. 48 h after seeding on filters, monolayers were tested for tightness using [^{3}H]inulin and conditioned medium was

assayed by ELISA after an additional 24 h. Control experiments indicated that monolayers were polarized by this time as judged by immunostaining using monoclonal antibodies to GP135 (gift of Dr. G. Ojakian, SUNY, Brooklyn), an apical marker, and E-cadherin (DECMA monoclonal, purchased from Sigma), a basolateral marker.

Immunoprecipitations and SDS-PAGE

Immunoprecipitations and 12.5% or 15% SDS-PAGE were performed as described previously (36) with the following modifications. Samples were incubated with rabbit NGF antisera (Sigma) overnight followed by a 2-h incubation with anti-rabbit IgG coupled to Sepharose (Sigma). Conditioned medium from metabolically labeled MDCK cells was analyzed in a similar manner.

RESULTS

Secretion of Neurotrophins by AtT-20 Cells

In order to examine the sorting of NGF, BDNF, and NT-3 into the regulated and constitutive secretory pathways, we expressed these neurotrophins in AtT-20 cells by stable and transient transfection. When the cells were transiently transfected with expression plasmids for NGF, BDNF, or NT-3, stimulation with a known secretagogue, 8-Br-cAMP, led to a 3-6-fold increase in the amount of all three neurotrophins detected within the conditioned medium as compared to unstimulated controls (Fig. 1*A*). Note that mock-transfected cells did not secrete significant levels of NGF, BDNF, or NT-3, indicating that there is little endogenous production of neurotrophins by AtT-20 cells.

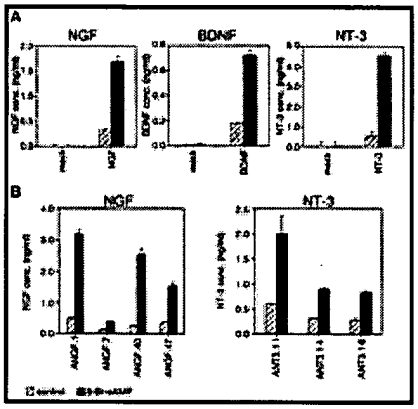

Fig. 1. Neurotrophin release by AtT-20 cells stimulated with 8-Br-cAMP. *A*, cells transiently transfected with the expression plasmids for NGF, BDNF, or NT-3 (NGF/pBJ5, BDNF/pBJ5, or NT3/pBJ5, respectively) were equally divided into six plates 24 h post tranfection. 72 h post transfection the cells were washed, and three plates were incubated in DMEM, 0.2% BSA containing 5 mM 8-Br-cAMP, a known secretagogue, while three served as unstimulated control. Cells were also transfected with vector alone (*mock*). The neurotrophin concentration within the conditioned media or in cell lysates was then assayed by ELISA. Data points represent average of three plates; *error bars* indicate S.E. *B*, AtT-20 cell lines were generated which stably expressed NGF or NT-3, and these cell lines were analyzed as described in *A*.
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Similar results were obtained when cell lines that stably expressed either NGF or NT-3 were analyzed (Fig. 1*B*) Four cell lines that expressed detectable levels of NGF, and three that expressed NT-3 were examined. In each case, stimulation with 8-Br-cAMP led to a 2.5-6-fold increase in the amount of neurotrophin released. There was a somewhat smaller increase in the release of neurotrophin after stimulation in NT-3 expressing lines as compared to those expressing NGF, suggesting that there may be differences in the efficiency with which NGF and NT-3 are sorted to the regulated pathway. This difference, however, was generally not observed in transiently transfected cells.

To verify that the 8-Br-cAMP-induced neurotrophin secretion was due to an increase in release from intracellular stores, as opposed to an increase in neurotrophin synthesis, we treated the cells with 100 μg/ml cycloheximide or 10 mM actinomycin D for 1.5 h prior to stimulation with 8-Br-cAMP and continued the treatment throughout the entire period of stimulation. Treatment of AtT-20 cells with this concentration of cycloheximide has been shown to inhibit 97% of new protein synthesis within 30 min (41). As shown in Fig. 2, 8-Br-cAMP induces a 4-fold increase in NGF secretion in the presence of cycloheximide. Similar results were obtained after treatment with actinomycin D, demonstrating that new mRNA or protein synthesis is not required for the secretagogue-induced NGF secretion. Interestingly, cycloheximide or actinomycin D treatment decreased NGF secretion by 8-Br-cAMP-stimulated cells but had little effect on unstimulated controls. This is consistent with the finding that cycloheximide treatment impaired regulated secretion to a greater extent than constitutive secretion in AtT-20 cells (41). Treatment with 8-Br-cAMP also led to significant decrease in the amount of NGF detected within cell lysates (Fig. 2). Together, these results provide evidence that the neurotrophins are released via the regulated secretory pathway by AtT-20 cells.

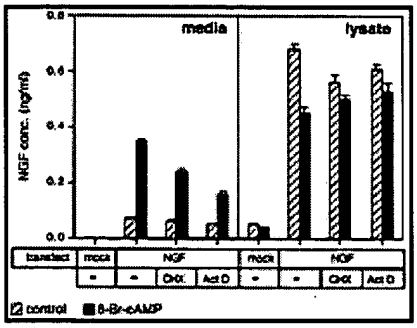

Fig. 2. Regulated release of neurotrophins from AtT-20 cells does not require new mRNA or protein synthesis. Cells transiently transfected with vector alone (*mock*) or NGF/pBJ5 were pretreated for 1.5 h with 100 μg/ml CHX, a protein synthesis inhibitor, or 10 mM actinomycin D (*Act D*), an mRNA synthesis inhibitor. Cells were then thoroughly washed and incubated for 4 h in DMEM, 0.2% BSA in the absence or presence of 5 mM 8-Br-cAMP. CHX or actinomycin D was present throughout the incubation period. At the end of the incubation period, cells were washed and lysed, and both lysate and conditioned media were assayed by ELISA. Data points represent average of three plates ± S.E.
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Regulated Neurotrophin Secretion by PC12 Cells

To determine if the neurotrophins were also released via the regulated secretory pathway in PC12 cells, cells were transiently transfected and analyzed as described for AtT-20 cells except that regulated secretion was stimulated by depolarization with 50 mM potassium chloride for 15 min. Similar conditions have previously been used to study the secretion of catecholamines, human growth hormone, and other molecules targeted to the regulated pathway in PC12 cells (42, 43). As seen in Fig. 3, depolarization with 50 mM KCl led to a 2.5-4-fold increase in the amount of NGF or NT-3 detected within the conditioned medium. This depolarization-induced release of NGF or NT-3 was not inhibited by cycloheximide or actinomycin D at concentrations that inhibit new protein or mRNA synthesis (44), indicating that this release was not due to new protein synthesis and supporting the conclusion that neurotrophin was being released from preexisting intracellular stores.

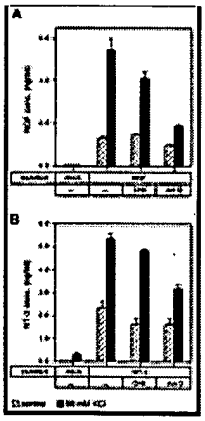

Fig. 3. PC12 cells transiently transfected with NGF/pBJ5 (*A*) or NT-3/pBJ5 (*B*) were pretreated with 100 μg/ml CHX or 10 mM actinomycin D (*Act D*) as described in the Fig. 2, then incubated in DMEM, 0.2% BSA with or without 50 mM KCl for 15 min. CHX or actinomycin D was present throughout the incubation period. Conditioned medium was subsequently assayed for NGF or NT-3 by ELISA. Data points represent average of three plates ± S.E.

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Sorting of NGF Propeptide Deletion Mutants

To investigate the mechanism of neurotrophin sorting and how it might be regulated, we used site-directed mutagenesis to examine regions or motifs within the NGF precursor that might be expected to influence the regulated secretion of NGF. Our analysis focused on the NGF propeptide for several reasons. First, this region contains potential sites for proteolytic processing and *N*-glycosylation, post-translational modifications that have been shown to play a role in the targeting of other proteins to specific secretory pathways (30, 32). Second, propeptides have themselves been demonstrated to carry critical sorting information (45, 46). Finally, based on our earlier studies, it was observed that mature, biologically active NGF could be produced even after deletion of more than 75% of the propeptide; by contrast, we find that relatively small deletions within the mature NGF moiety can drastically destabilize the protein.[2] Three groups of proNGF mutants were analyzed (Fig. 4). In the first group, the propeptide was divided into five regions and each of these deleted individually. Expression of these mutants in COS cells has already been described (18). In the second group, three potential processing sites were altered. In the third group, two potential *N*-glycosylation sites were mutated either alone or in combination. A mutant lacking residues in the amino terminus of mature NGF (NGFΔ3-8) (47) was also analyzed.

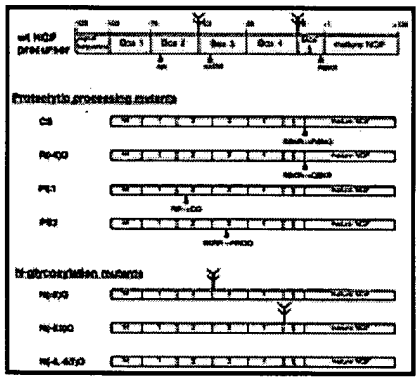

Fig. 4. Schematic diagram of NGF propeptide mutants. Note that mature NGF is not drawn to scale. Constructs were created by polymerase chain reaction double overlap mutagenesis as described under ``Materials and Methods.'' Construction and expression of R(−4)Q and N(−8)Q mutants has been described previously (18, 36).

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When the propeptide deletion mutants were expressed in AtT-20 cells NGF was consistently detected in the conditioned medium of cells transfected with the Δ1, Δ2, Δ4, Δ5, and NGF(Δ3-8) mutants (although the relative expression levels of these mutants varied) but never after transfection with the Δ3 mutant. When regulated secretion was assessed as described above, stimulation with 8-Br-cAMP led to at least a 3-fold increase in NGF secretion for all of the detectable mutants (Fig. 6*A*). The lower levels of NGF secretion for some mutants was not due to their selective retention within the cell, as NGF levels were

lower within the cell lysates of these mutants as well (Fig. 6*A*). These data suggest that information required for regulated secretion is not contained within regions 1, 2, 4, or 5 of the NGF propeptide or within the amino terminus of mature NGF. Similar results were obtained for a mutant lacking the three carboxyl-terminal residues of mature NGF (data not shown).

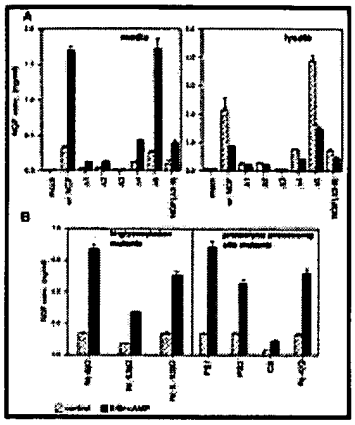

Fig. 6. Secretion of NGF propeptide deletion mutants (*A*), *N*-glycosylation mutants, and proteolytic processing mutants (*B*) by AtT-20 cells. Deletion mutants were transiently expressed in AtT-20 cells and analyzed as described in Fig. 1. To ensure that lower expressed deletion mutants were not retained within the cell, lysates from the same samples were also assayed by ELISA. Data points represent average of three plates ± S.E.

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The lack of expression of the Δ3 mutant is consistent with our earlier demonstration that only region 3 of the propeptide was strictly required for NGF production in COS cells. When this region was deleted, NGF precursor appeared to be rapidly degraded within the endoplasmic reticulum (18),[3] suggesting that it is involved in the proper folding or dimerization of the nascent NGF precursor.

The Role of Proteolytic Processing in Regulated Secretion

The NGF propeptide contains one tetrabasic and two dibasic sites, each of which appears to be cleaved *in vitro* (48). Furin, and possibly other prohormone convertases, cleave at the third of these sites to liberate mature NGF (49, 50). We used site-directed mutagenesis to eliminate each of these sites individually (PS1, PS2, and CS mutants; see Fig. 4). We also analyzed a mutant (R(−4)Q) in which the amino-terminal arginine within the R*X*(K/R)R consensus recognition sequence was altered but the dibasic site left intact (36). When expressed in COS cells, the CS and R(−4)Q mutants were secreted as uncleaved proNGF (Fig. 5*A*), which was confirmed by immunoprecipitation with an antibody directed against a region near the amino terminus of the NGF propeptide (51). The PS1 and PS2 mutants were expressed and processed to mature NGF in a manner indistinguishable from wild type NGF (data not shown). When these mutants were transiently expressed in AtT-20 cells, NGF secretion was stimulated by 8-Br-cAMP to a similar extent as in the case of wild-type NGF (Fig. 6*B*) suggesting that proteolytic processing at dibasic or tetrabasic sites within the NGF precursor is not required for regulated secretion. Because expression of these mutants was too low to detect by metabolic labeling and SDS-PAGE, however, we were not able to rule out the possibility that cleavage occurs at other sites within the propeptide when the dibasic sites are mutated.

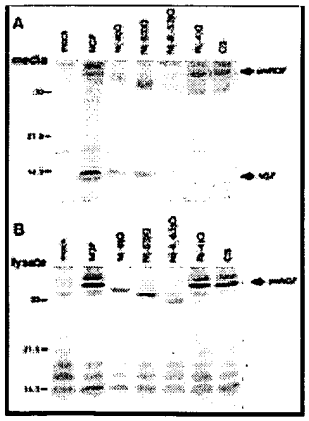

Fig. 5. NGF expression of propeptide mutants in COS cells. *A*, analysis of secreted proteins. COS cells transiently transfected with mutants described in Fig. 4 were metabolically labeled for 3 h with [^{35}S]cysteine. Conditioned medium was collected, immunoprecipitated with NGF antisera, and analyzed by 12.5% SDS-PAGE. *B*, metabolically labeled cell lysates from the same samples were subjected to a similar analysis as described in *A*.

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The Role of N-linked Glycosylation in Regulated NGF Secretion

The NGF propeptide contains two consensus *N*-glycosylation sequences. One of these sequences, located 8 amino acids upstream of mature NGF, is conserved in all known neurotrophin precursors. Earlier work demonstrated that the precursor was *N*-glycosylated at least at this conserved site (17, 18). To study the role of *N*-glycosylation in NGF sorting, we analyzed mutants in which either or both of the acceptor asparagines was changed to glutamine (Fig. 4). When these mutants were expressed in COS cells, mature, biologically active NGF was secreted at levels consistently lower than that of wild-type NGF (Fig. 5*A*). To verify that both of these sites were used, the lysate of transfected; metabolically labeled cells was immunoprecipitated with NGF antisera followed by SDS-PAGE; as seen in Fig. 5*B*, the apparent size of the precursor was reduced when either site is mutated, and is further reduced when both are mutated, providing evidence that both sites were used. When these mutants were transiently transfected into AtT-20 cells, NGF release was stimulated 3-6-fold by 8-Br-cAMP (Fig. 6*B*) demonstrating that *N*-glycosylation of the NGF precursor is not required for sorting to the regulated secretory pathway.

Neurotrophin Secretion by MDCK Cells

To investigate neurotrophin targeting in polarized cells, stably or transiently transfected MDCK cells were grown on semipermeable filters as described previously (37). After 3-4 days the filters were washed and fresh media added to both the apical and basolateral compartments. Medium was then collected after 24 h and assayed for neurotrophin content by ELISA. Control experiments demonstrated that, within 48 h after seeding, both transiently and stably transfected cells were able to form tight monolayers and target proteins correctly as demonstrated by the polarized staining of GP114, an apical marker, and E-cadherin, a basolateral marker (see ``Materials and Methods" for details). As seen in Fig. 7*A*, when two stable cell lines were analyzed, MNGF.2 and MNGF.5, 65-69% of the total secreted NGF was detected on the basolateral side. A similar percentage was also detected in transiently transfected MDCK cells and in a pool of more than 40 stable clones, indicating that these results were not due to clonal variation (data not shown). To examine the possibility that the targeting of NGF could be regulated by alternative splicing of the NGF gene, we analyzed stable cell lines (MLNGF.4 and MLNGF.5) transfected with the cDNA encoding the ``long" NGF precursor, an isoform arising from alternative splicing of the NGF gene (52). The polarity of NGF secretion in these cell lines did not differ significantly from other NGF-expressing cells (Fig. 7*A*).

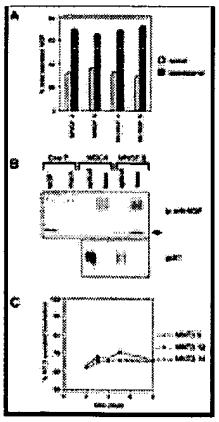

Fig. 7. Neurotrophin secretion by MDCK cells. *A*, stable cell lines were generated using expression plasmids for NGF (*MNGF.4* and *MNGF.5*) or the alternatively spliced long NGF precursor (*MLNGF.4* and *MLNGF.5*). Cells were then grown as confluent monolayers on 24.5-mm collagen-coated Transwell filters (0.4-μm pore size) for 3-4 days. The monolayers were washed, and fresh medium was added. Medium was collected from apical and basolateral compartments after 24 h and assayed for NGF content by ELISA. For each cell line three filters were analyzed; data represent average of three filters ± S.E. *B*, parental MDCK cells or MNGF.5 cell line were grown on filters as described in *A*, then metabolically labeled for 3 h with [^{35}S]cysteine (*top panel*). Medium was immunoprecipitated with NGF antisera (*top panel*) and analyzed by 12.5% SDS-PAGE. gp81, an apically secreted endogenous protein, was analyzed under nonreducing conditions as described previously (38). *C*, NT-3-expressing stable cell lines were generated and analyzed as described in *A*, except that medium was collected every 24 h and assayed. Three filters were grown for each of the three cell lines. Each data point represents the average of three filters ± S.E. For points with no *error bars*, S.E. was less than 1%.
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To verify the polarity of NGF secretion, the cell line MNGF.5 was metabolically labeled for 3 h and media from the apical and basolateral compartments were immunoprecipitated with NGF antisera followed by SDS-PAGE (Fig. 7*B*). MNGF.5, but not the parental MDCK line, secreted a product which appears to be mature NGF based on its electrophoretic mobility and ability to be immunoprecipitated by NGF antisera. 71% of this product was detected basolaterally as determined by densitometry, in agreement with the ELISA results. MNGF.5 retained its ability to target proteins to the apical membrane as evidenced by the secretion of gp81, an endogenous glycoprotein (39, 40). BDNF, which was tested only by transient transfection, was secreted 71 ± 0.5% basolaterally (n = 3). Similarly, three cell lines stably expressing NT-3 all secreted 65-70% basolaterally. This percentage was reached by the 3rd day on filters and remained stable through the 8th day (Fig. 7*C*). Thus it appears that NGF, BDNF, and NT-3 are secreted with a similar polarity by MDCK cells.

DISCUSSION

In this study we have expressed NGF, BDNF, and NT-3 in neuroendocrine cells containing a regulated secretory pathway and in polarized epithelial cells in order to assess how the neurotrophins are sorted and secreted and whether there are differences between the neurotrophins that could be physiologically significant. Furthermore, we have used site-directed mutagenesis to examine the molecular determinants of NGF sorting to the regulated secretory pathway. We observed that NGF, BDNF, and NT-3 were all secreted by the regulated pathway when expressed in AtT-20 and PC12 cells, with a 3-6-fold increase consistently detected in the conditioned media after stimulation. Regulated neurotrophin secretion was observed after transient transfection as well as in stable cell lines with widely varying levels of neurotrophin expression and is therefore unlikely to be an artifact caused by clonal variation or expression level. The NGF findings are consistent with earlier studies (17, 19), and, taken together with our data for BDNF and NT-3, suggest that the neurotrophins are sorted similarly by a mechanism which functions in diverse cell types.

For some precursors it is known that sorting information may be contained within the propeptide and the specific cleavage sites used within the precursor can regulate this sorting (31, 32). To determine if the

NGF propeptide influenced sorting, we analyzed mutants containing deletions which scanned the entire propeptide. Each of these mutants except one (Δ3 mutant) was expressed at detectable levels and underwent regulated release. Mutants in which the consensus *N*-glycosylation acceptor sites or potential proteolytic processing sites were abolished were also secreted by the regulated pathway. Therefore, for NGF, and presumably the other neurotrophins as well, the signals required for sorting to the regulated pathway are likely to lie within the mature neurotrophin. This is reasonable in light of the fact that mature neurotrophins are highly conserved, sharing ~50% amino acid identity, while their propeptides are much less related (53, 54). The nature of the sorting ``signal'' remains to be determined; it may be a linear stretch of amino acids, a structural motif, or a property of the protein such as its net charge (see below).

What is the mechanism by which neurotrophins are directed to the regulated pathway? According to two current models, regulated and constitutive proteins may be segregated on the basis of their affinity for a receptor or, alternatively, by their ability to multimerize or aggregate in the conditions within the trans-Gogi network (55, 56). Our data do not directly discriminate between these models. However, while it is known that PC12 cells have both trkA receptors (which bind NGF and to a lesser extent NT-3) and p75 (which binds all known neurotrophins), but not trkB or trkC, none of the known neurotrophin receptors were detected in AtT-20 cells by immunoblotting (data not shown). Since both cell types sort the neurotrophins to the regulated pathway, we conclude that the known neurotrophin receptors are not required for this sorting. It is possible that the sorting is mediated by low affinity interactions between neurotrophins and molecules within the regulated secretory pathway. For example, the strong positive charge on the neurotrophins (all have pI values greater than 9.0) may promote interactions with negatively charged molecules such as secretogranins, a family of acidic proteins that are believed to act as helper proteins in the sorting of peptide precursors to the regulated secretory pathway. One member of this family, chromogranin B, increases the efficiency of ACTH sorting when overexpressed in AtT-20 cells (57). Interestingly, when either chromogranin B or secretogranin II is coexpressed with NGF in AtT-20 cells the processing of NGF is altered, suggesting that the secretogranins interact with either NGF or a processing enzyme (50). It would be interesting to determine if the efficiency of NGF sorting to the regulated pathway is increased by secretogranin coexpression. Finally, while it is not known if the neurotrophins undergo a milieu-induced multimerization under trans-Gogi network conditions, sedimentation equilibrium data for NGF, BDNF, and NT-3 suggest that all three form some higher order oligomers at pH 7.1 (58).

Neurotrophin Secretion by MDCK Cells

We find that NGF, BDNF, and NT-3 were all secreted with a similar polarity by MDCK cells, with 65-75% of the total neurotrophin detected in the basolateral compartment. The alternatively spliced ``long'' NGF precursor was secreted identically as the shorter form, a finding consistent with the earlier observation that both forms were sorted to the regulated pathway in AtT-20 cells (17). The secretion of the neurotrophins is considerably less polarized than the greater than 90% basolateral secretion observed for the endogenous basement membrane components laminin and heparin sulfate proteoglycan (59) and only slightly more than the 60-65% observed for other transfected proteins such as growth hormone, chicken oviduct lysozyme, and cystatin C (40, 60), which are all sorted to the regulated pathway in AtT-20 cells but are believed to lack signals for polarized sorting and be secreted by the bulk flow ``default'' pathway in MDCK cells. The neurotrophin results could therefore be explained by either active but relatively inefficient basolateral sorting or by the absence of epithelial targeting signals.

Implications for Neuronal Sorting of Neurotrophins

Neurons are unique in their morphological and functional complexity and it is unlikely that any cell line

can provide a exact model for all the diverse mechanisms that neurons employ to target proteins. In fact, there are substantial differences even between different types of neurons (61). Nevertheless, the existing data suggest that neurons mainly rely on mechanisms also found in non-neuronal cells (24). In particular, there appear to be strong similarities in the exocytotic machinery and sorting mechanisms that neurons, neuroendocrine, and endocrine cells employ for regulated protein and peptide release (23). The earlier findings that NGF undergoes regulated secretion when heterologously expressed in endocrine and hippocampal neurons support this conclusion (17, 19). Based on these earlier studies and our own data, we predict that BDNF and NT-3, in addition to NGF, undergo regulated secretion in neurons.

Our findings in MDCK cells suggest that the secretion of NGF, BDNF, and NT-3 is more somatodendric than axonal but not highly polarized. This is consistent with immunocytochemical studies of BDNF localization in the hippocampus and cortex (13, 62). However, the MDCK data should be interpreted cautiously. The model that the axonal and dendritic domains of neurons are equivalent to the apical and basolateral domains, respectively, of epithelial cells is based on the localization of transmembrane proteins such as transferrin receptor, vesicular stomatitis virus G protein, viral hemagglutinin protein, and the γ-aminobutyric acid transporter GAT-1, as well as glycosyl phosphatidylinositol-linked proteins such as Thy-1 (reviewed in Refs. 61 and 63); the parallel between neuronal and epithelial sorting has not proven to be absolute (61, 63) and has not been established for secreted proteins. A critical question which remains unresolved is whether protein targeting to different subcellular locations in the neuron (*i.e.* axon *versus* dendrite) and sorting to the regulated *versus* constitutive pathways are determined independently. In some cell types regulated secretion is confined to a single domain (*i.e.* the apical membrane of exocrine cells). Indeed, it has been suggested that regulated and constitutive neurotrophin secretion occur at the dendrites and cell body, respectively (8). In general terms, protein targeting can be divided into two steps: the sorting of proteins into particular organelles, and the targeting of these organelles to their cellular destinations (24). It may prove to be the case that the first step is well conserved for neurotrophins among diverse cell types, while the second step is more dependent on factors such as the developmental stage, cytoarchitecture, and environment of a given cell.

In conclusion, our data suggest that all the neurotrophins are capable of undergoing regulated secretion and suggest that the signal(s) for sorting to this pathway lie within the mature neurotrophin moiety. Important questions about neurotrophin secretion remain to be answered. For example, can regulated neurotrophin secretion occur locally, *i.e.* at a single synapse or dendrite, and therefore serve as a means to specifically reward active connections? Can a neuron regulate the targeting of neurotrophins by other mechanisms such as mRNA targeting or by expressing accessory molecules which direct these proteins to specific subcellular locations or secretory pathways? Finally, in what type of vesicles are neurotrophins stored and what stimuli trigger their release? Understanding these and other issues surrounding neurotrophin targeting will help clarify the role of the neurotrophins in the development and plasticity of the nervous system.

FOOTNOTES

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[1] The abbreviations used are: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT, neurotrophin; MDCK, Madin-Darby canine kidney; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; CHX, cycloheximide; PAGE, polyacrylamide gel electrophoresis; Br, bromo.

[2] A. Krüttgen, J. V. Heymach, Jr., P. Kahle, and E. M. Shooter, unpublished observations.

[3] J. V. Heymach, Jr., A, Krüttgen, U. Suter, and E. M. Shooter, unpublished observations.

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